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MIXING CONSIDERATIONS IN THE DEVELOPMENT OF A GRADIENT MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM

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SUMMARY

A home-made gradient microbore high-performance liquid chromatographic (HPLC) system was designed using two Varian 8500 stepper motor-driven syringe pumps. The pump delivery of the mobile phase solvents in the 10 μl/min flow-rate range is quite pulsed in nature. Static and dynamic mixing devices were evaluated in terms of their ability to homogenize the slugs of solvent delivered by the pumps. Poor mixing was obtained when a simple tee was used to combine the solvents; this incomplete mixing resulted in increased baseline noise, poorer retention time reproducibility, and a significant decrease in column plate count. The use of static mixing tubes did not yield a completely homogenized mobile phase. A stirred mixing chamber with an internal volume of 80 μl gave the best results, and chromatographic performance was not degraded through use of this mixer. Stirred mixing chambers do distort the linearity of a gradient through exponential dilution; a computer simulation of a stirred mixing chamber was developed in order to study the dependency of exponential dilution effects upon mixing-chamber volume, ramp-rate, and flow-rate. A simple formula was obtained that allows one to use these variables to calculate the extent of gradient distortion. Finally, comparison of gradient microbore and conventional HPLC for polymer fractionations showed that the two methods performed equally well.

INTRODUCTION

The use of microbore (1 mm I.D. or less) columns in high-performance liquid chromatography (HPLC) has become an accepted technique. Some advantages of these columns as compared to conventional bore (3–5 mm I.D.) columns include lower mobile phase solvent consumption, lower amounts of stationary phase required to pack the column, the ability to use small (sub-μl) amounts of sample, and inher-

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ently higher sensitivity of the technique since less dilution of the sample by the mobile phase occurs[1-3]. The development of microbore HPLC systems was pioneered by Ishii et al.[4] and by Kucera and co-workers[5-8]. Typical columns are 15 to 50 cm long with a 1 mm I.D., and flow-rates are 10–50 μl/min. Injector and detector volumes are typically 0.1–1 μl, as extra-column dead volume must be kept to a minimum to reduce band broadening.

Techniques of gradient elution in microbore HPLC were not developed as rapidly as the miniaturization of injectors, columns, and detectors occurred. Microbore flow-rates of 10–50 μl/min either cannot be delivered by most commercial HPLC pumps or are at the extreme lower end of their working range. Gradient elution at microbore flow-rates with these pumps is therefore impossible. However, some of these pumps can be readily modified to deliver lower flow-rates, and pumps specially designed for microbore work are now commercially available. Most gradient microbore HPLC to date has utilized the technique of high-pressure mixing[9,10] where two (or more) pumps deliver the component solvents to a mixing chamber. The mixing chambers (volume: 1–2 ml) used to homogenize the pump effluents in conventional HPLC are not suitable for microbore work because the resulting exponential dilution will distort the linearity of the gradient[11].

Several methods of obtaining gradients at microbore flow-rates are reported in the literature. Initially, gradients for microbore work were generated by a special apparatus external to the HPLC system and stored in a delivery tube for use in the actual run[4]. Gradients were generated on-line by Scott and Kucera[12] using a mixing tee and two fairly pulse-free pumps, with satisfactory results. Takeuchi and Ishii[13,14] used a micromixer with a volume of about 100 μl to generate an exponential gradient for capillary HPLC[15], where flow-rates are in the 1–10-μl/min range. Linear gradients for capillary HPLC were developed by Van der Wal and Yang[16], who generated gradients at conventional flow-rates and split 1% of the flow to the capillary column. The advantage of low solvent consumption is lost through use of this technique. Static mixers are also becoming popular in conventional and microbore HPLC because of their simplicity; a laminar capillary mixer, which is simply a flattened length of tubing, is featured on some commercially available systems that can be used for conventional and micro-HPLC[11]. A recently introduced solvent delivery system for microbore HPLC utilizes columns packed with glass beads as static mixers[28].

The first comparative study of dynamic (stirred) and static mixers was recently carried out by Schwartz et al.[17]. Relatively pulse-free reciprocating pumps were used, and the study emphasized the dependence of the detector baseline noise upon the quality of mixing. It was observed that the use of a low volume tee as the sole mixing device resulted in the most baseline noise, whereas a stirred mixing chamber with a volume of 35 μl gave the least noise. State mixers consisting of various lengths of 1 mm I.D. empty tubing gave intermediate results. Distortion of the overall linearity of the gradient was most significant when a dynamic mixer was used, whereas use of static mixing tubes simply delayed the appearance of the gradient at the column inlet by time intervals proportional to the length of the tube.

This study confirms the relative efficiencies of different methods of mixing, and it will focus on the use of two stepper motor-driven syringe pumps to deliver the strong and weak solvents during the course of the gradient. Syringe pumps have been used in microbore[18] and capillary[19,20] HPLC for isocratic work. The stepper motors

operate at low frequencies (0–3 Hz) during the course of a microbore gradient, so the solvents are delivered in discrete pulses. Poor mixing resulted in increased baseline noise and decreased retention time reproducibility, as expected[17]. An additional unexpected effect was a dramatic increase in band broadening. Therefore, an efficient, low-volume mixing device is essential. When various static and dynamic mixers were evaluated, the best results were obtained by using a stirred mixing chamber with a volume of 80 μl. The performance of the system under gradient conditions is comparable to that obtained in conventional HPLC.

The main disadvantage of using dynamic mixing chambers is that the linearity of the gradient is distorted through exponential dilution. A computer simulation was developed in order to study the effects of ramp-rate, flow-rate, and mixing chamber volume upon the gradient linearity. A simple formula was obtained that relates these parameters, and can be used to predict the linear range of a gradient generated using a stirred mixing chamber under a wide range of HPLC conditions.

EXPERIMENTAL

Major equipment

Solvent delivery system. Two Varian 8500 syringe pumps with a gradient controller (Varian Instruments Division, Sunnyvale, CA, U.S.A.) were used to deliver the strong and weak solvents. The gradient controller was electronically modified to bring microbore flow-rates (10–50 μl/min) into the working range of the system electronics. This was accomplished by inserting a divide-by-ten counter after the output of the flow-rate controller, which is a transistor–transistor logic (TTL)-level pulse train whose frequency (in Hz) is equal to the total flow-rate (in ml/h)[21]. The output of this counter (flow-rate controller frequency/10) was then fed into the circuitry that controls the solvent composition. This modification permits 30 choices of flow-rates between 0 and 50 μl/min instead of the three that would be obtained with the unmodified circuitry. The divide-by-ten counter was bypassed when conventional gradient HPLC runs were to be made for comparison to the microbore results.

Mixing devices. The simplest device used to combine the two pump effluents was a mixing tee (part no. ZT1C, Valco Instruments, Houston, TX, U.S.A.) with a 0.25-mm bore. If static mixing was desired, the mixing tee was followed by a piece of empty stainless-steel tubing (1/16 in. O.D., 1 mm I.D.). Static mixing tubes with volumes of 80 μl (9.9 cm in length) and 320 μl (39.5 cm) were evaluated. The dynamic (stirred) mixer was custom-designed and a cross-section of it is shown in Fig. 1. The internal volume (with the stirbar in place) is 80 μl. Both solvents are delivered at the bottom and the resulting mixture is forced out at the top to maximize the residence
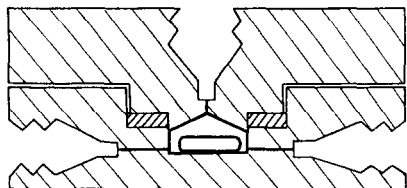

Fig. 1. Cross section of 80-μl stirred mixing chamber.

time of the solvent slugs delivered by the pumps. The top is conical-shaped to facilitate removal of trapped air and to minimize unswept volume in the mixing chamber. A magnetic stirbar (1 mm thick and 3 mm long, Lab Glass, Vineland, NJ, U.S.A.) was used in the chamber, and the mixing chamber was bolted to a small magnetic stirrer (Model 120 MR, Fisher Scientific, Norcross, GA, U.S.A.). An 8 cm × 0.13 mm I.D. tubing (internal volume of 1.0 μl) was used to connect the mixing chamber to the injector.

Air bubbles were often observed to emit from the mixing chamber when solvents of different polarities (such as methanol and water) were combined. Thorough degassing and addition of methanol (10–30%, v/v) to the water beforehand did not solve this problem. The best solution was to combine the two pump outlets with a mixing tee, then connect the outlet of the tee to one of the mixing chamber inlets with a 9.9 cm × 1 mm I.D. (80 μl internal volume) empty tubing. The other mixing chamber inlet was plugged. With this modification, the two solvents were fairly well-mixed before they entered the mixing chamber, and air bubbles did not have a tendency to form. In some cases, such as when acetonitrile and water were used, it was necessary to pack the premixer tube with 0.45 mm diameter glass beads (B. Braun Melsungen, Ternruf, F.R.G.). The use of glass beads[28] improved the homogeneity of the solution entering the mixing chamber enough to eliminate air bubble formation in all cases studied.

Other LC hardware. An electronically-actuated injector valve (Model EC14W, Valco Instruments) with an internal 0.5-μl sample loop was used to deliver the sample to the column. The column was connected directly to the injector and to the detector flow-cell in order to minimize extra-column dead volume. A variable-wavelength UV detector (Model SF769Z, Kratos Instruments, Ramsey, NJ, U.S.A.) was used to monitor absorbance at 254 nm. The flow-cell had an internal volume of 0.5 μl. A stripchart recorder (Model 585, Linear Instruments, Irvine, CA, U.S.A.) was used to record the absorbance data.

Reagents. Methanol and acetonitrile (HPLC grade, J. T. Baker, Phillipsburg, NJ, U.S.A.) were used without further purification. Doubly-distilled de-ionized water was prepared from house distilled water in our laboratory using a home-made purification system consisting of anion and cation exchangers, an activated charcoal bed, and a distillation apparatus. Polystyrene (MW 666) was obtained from Arro Labs (Joliet, IL, U.S.A.).

HPLC columns

Microbore HPLC columns were packed in our laboratories using a slurry packing procedure. Tubing (1/16 in. O.D., 1 mm I.D., Alltech, Norcross, GA, U.S.A.) was cut into 50-cm pieces. It has been reported[22] that the inner wall of the tubing must be smooth in order to pack a good microbore column. Therefore, the inner bore was polished by pumping a slurry of Carborundum 600 polishing compound in water through the tubing using a recirculating peristaltic pump for 24 h. The column packing material was Zorbax 5-μm octadecylsilyl-derivatized spherical silica (DuPont Instruments, Glasgow, DE, U.S.A.).

The packing procedure is similar to that reported by Siemion[23]: the column was filled with tetrachloromethane before packing was started, and a slurry of the packing material (10%, w/v) in isopropanol–cyclohexanol (1:3, v/v) was poured into

the slurry bomb. A constant-pressure air-driven reciprocating pump (Model 10-600-50, SC Hydraulics, Los Angeles, CA, U.S.A.) was used to force methanol through the column at a pressure that was increased from 15,000 to 20,000 psi during the first minute of packing. We and Bouwermaster and McNair[22] have observed that this type of pressure increase during the early stage of column packing gives the best results. Pure methanol was allowed to pass through the column for 30 min, after which the "slamming" technique[24], in which the column is allowed to depressurize and then is rapidly repressurized, was used several times to consolidate the packed bed. The column was connected directly to the injector port, which contained a 2-μm porosity, 1/16 in. diameter screen (part no. 2SR1, Valco Instruments) to retain the packing in case of a sudden inlet pressure drop. A 1/16 in. diameter, 1/32 in. thick, 0.5-μm porosity frit (Alltech Associates) was used in the detector flowcell inlet port.

The conventional HPLC column used in this study was a 1/4 in. O.D., 25 cm × 4.6 mm I.D. column obtained from IBM Instruments (Danbury, CT, U.S.A.). The column packing was 5-μm octadecylsilyl-derivatized spherical silica.

Calculations and simulations

Chromatographic calculations. Retention times, linear velocities, and HETP values were determined in the usual manner. Plate counts were obtained by using the peak width at half-height.

Computer simulations. Computer simulations of gradients produced using a stirred mixing chamber were performed on a PDP 11/23 computer (Digital Equipment, Maynard, MA, U.S.A.); all programming was done in RSX-11M version 3.2 FORTRAN. The model assumed an expandable mixing chamber of volume V containing a strong solvent concentration C_{old}. A volume (Δ) of new solution having a strong solvent concentration $C_{in.}$ was then added to the mixing chamber, which caused it to "swell" to a volume of $V + \Delta$. Perfect mixing was assumed, and the new concentration in the mixing chamber, C_{new}, is given by:

$$C_{new} = (C_{in} + C_{old}V)/(V + \Delta) \qquad (1)$$

The mixing chamber is then "deflated" back to a volume V by delivering a slug of concentration C_{new} and volume Δ. The value of C_{old} was replaced by the value of C_{new} and the process repeated. This model approaches reality if the slug volume is 1–2% of the mixing chamber volume and the concentration C_{in} is updated by small increments several thousand times during the course of the gradient. The program allows the operator to simulate gradients under a variety of flow-rate, ramp-rate and mixing chamber volume conditions; the starting point of the gradient is also freely variable.

RESULTS AND DISCUSSION ·

Evaluations of different mixing devices

Test gradients. The linearity and smoothness of gradients produced using the various mixing devices was studied by having the "weak solution" pump deliver pure methanol and the "strong solution" pump deliver methanol spiked with acetone. The

outlet of the mixing device being studied was connected directly to the detector flow cell with an 8 cm × 0.13 mm I.D. tubing, which produced a negligible delay volume of 1.0 μl. The acetone absorbance was monitored at 254 nm during the course of the gradient. All gradients covered the 0–100% methanol to acetone–methanol range in 50 min at a rate of 2%/min, with a flow-rate of 50 μl/min. The gradients obtained with the tee, 80 and 320 μl static mixing tubes, the 80 μl stirred mixing chamber, and the tee to 80 μl static mixing tube to 80 μl stirred mixing chamber combination are shown in Fig. 2A–E.
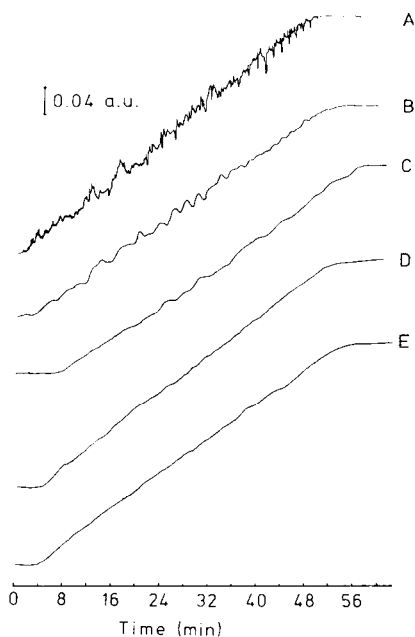

Fig. 2. Test gradients obtained with methanol and methanol spiked with acetone. A, Mixing tee only; B, mixing tee with 80-μl static mixing tube; C, mixing tee with 320-μl static mixing tube; D, 80-μl stirred mixing chamber; E, mixing tee, 80-μl static mixing tube and 80-μl stirred mixing chamber.

The overall linearity of the gradient obtained with the mixing tee (Fig. 2A) was quite good, but the short-term noise corresponded to absolute concentration fluctuations of *ca.* 7–8%. The noise was present because the two pumps delivered their solutions in short bursts; the stepper motors driving these pumps operated at frequencies of only 0–3 Hz at a total combined flow-rate of 50 μl/min. Homogenization of these pulses was far from complete in the mixing tee. Pulse damping was not a feasable alternative; the duty cycle of the pulse train was too low, and we have noticed, along with Scott and Kucera[12], that at low flow-rates a solvent delivered by one pump can accumulate in the pulse damper of the other pump.

Addition of a static mixing tube to the tee did smooth out the short-term noise to a significant extent. The gradient produced using the 80-μl static mixer (Fig. 2B) was not smooth enough, but the quality was almost adequate when the volume was quadrupled to 320 μl (Fig. 2C). However, notice that the appearance of the gradient

at the detector was delayed by 7–8 min for the longer mixing tube. Clearly, a static mixing tube long enough to remove all oscillations would yield delay volumes[17] that are much too large. The overall linearity of the gradient appears to be quite good, and this feature, along with the inherent mechanical simplicity of static mixers, has made them quite popular in present-day commercial instrumentation where virtually pulse-free pumps are used[11,28]. The significant pulsing nature of the syringe pumps ruled out the possibility of static mixing in this study.

The best performance, in terms of delay volumes and smoothness, was obtained when the 80-μl dynamic mixer was used (Fig. 2D). The addition of the tee and 80-μl static tube to the mixing chamber did not seem to affect the linearity of the gradient (Fig. 2E). However, the starting point of the gradient was delayed by about 1.5 min, as was observed for the 80-μl static mixer alone. The gradient looks very similar to those obtained in conventional HPLC. There was a slight amount of rounding at the beginning and end of the gradient due to exponential dilution. However, exponential dilution did not produce serious distortion in the set of conditions used in this study. The results of further studies of exponential dilution are presented in a later section of this paper.

There was still some low frequency noise present in the gradients obtained using the stirred mixer. This noise was probably caused by the flow-rates of the two pumps drifting with respect to one another during the course of the gradient. The magnitude of the resulting variations in mobile phase composition was measured by holding the methanol to acetone–methanol ratio constant at 30:70 and observing the detector signal. The drift in the signal corresponded to fluctuations of *ca.* 0.5% in the mobile phase composition.

Isocratic separations. The effects of mixing upon chromatographic performance were evaluated using isocratic elution of a mixture of benzene derivatives. A methanol–water (70:30, v/v) mobile phase was used to separate uracil, phenol, acetophenone, nitrobenzene, methyl benzoate and toluene. The optimum chromatographic performance, in terms of baseline noise, retention time reproducibility, and plate count was determined when one pump was used to deliver a premixed methanol–water (70:30, v/v) mobile phase at a flow-rate of 50 μl/min (Fig. 3A). These results are compared to those obtained using both pumps to deliver methanol and water separately, but in the same ratio, to the mixing tee (Fig. 3B), and to the combination of the tee, 80-μl static mixing tube, and 80-μl stirred mixing chamber (Fig. 3C).

First, it is obvious that the use of the mixing tee alone resulted in increased baseline noise in agreement with results obtained by Schwartz *et al.*[17]. However, the noise increase in our work was much greater, due to our use of pumps having significant pulsations. Also, the poor mixing resulted in significant band broadening. Toluene, the most strongly retained component of the test mixture, yielded a plate count of only 4300, compared to the 11,000 theoretical plates obtained with the premixed mobile phase. This decrease of 61% indicates that poor mixing of the mobile phase component solvents can significantly degrade the performance of a good column. The toluene peak even developed some shoulders, presumably due to the inhomogeneity of the mobile phase. The reproducibility of the retention time for toluene decreased from 0.4% relative standard deviation (R.S.D.) using the premixed mobile phase to 4.0% R.S.D. using the mixing tee alone to combine the solvents.
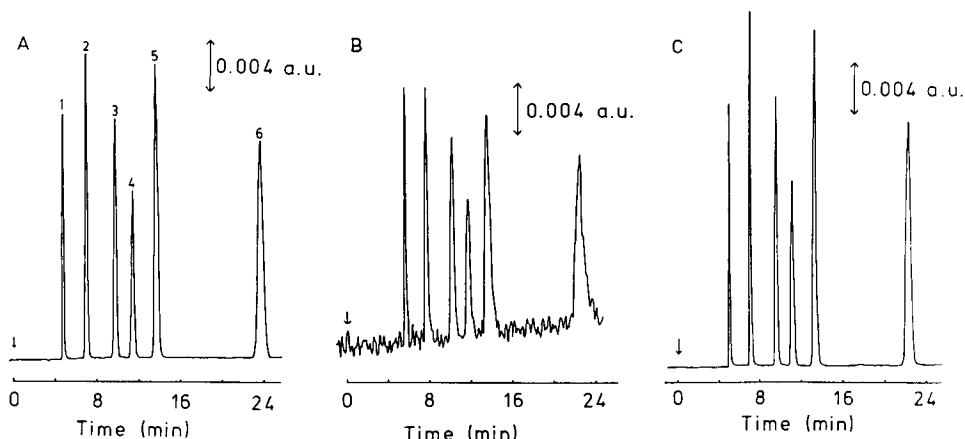
Fig. 3. Isocratic separations with a methanol–water (70:30, v/v) mobile phase. Peaks: 1 = uracil, 2 = phenol, 3 = acetophenone, 4 = nitrobenzene, 5 = methylbenzoate, 6 = toluene. (A) Premixed mobile phase delivered with one pump. (B) Methanol and water delivered with two pumps and combined with mixing tee. (C) Methanol and water delivered with two pumps and mixed with combined mixing devices.

The more efficient mixing obtained with the combined mixing devices improved the chromatographic performance. A comparison of Figs. 3A and 3C shows that the baseline noise and peak widths were about equal for premixed and on-line mixed mobile phases. Therefore, the mixing obtained using the combined mixing devices was good enough to yield optimum chromatographic performance. The retention t:ime of toluene had a 1.2% R.S.D. This increase in R.S.D. over the case where a premixed mobile phase was used was probably caused by drift of the flow-rates of the two pumps with respect to one other. As noted above, this drift caused a fluctuation of *ca.* 0.5% in the mobile phase composition, and the retention times in this separation are particularly sensitive to variations in solvent strength.

The efficiency of the column was evaluated over the 10–50 μl/min flow-rate range for the premixed and on-line mixed mobile phases. On-line mixing was done using the three combined mixing devices. The plate heights for toluene are plotted against the linear velocity in Fig. 4. The minimum plate height was achieved at a flow-rate of 20 μl/min, and had a value of 27.2 μm for the premixed mobile phase and 25.0 μm for the on-line mixers. Consistently lower plate heights were obtained when the on-line mixers were used. This result is quite surprising, since one would have expected the best mobile phase mixing to have been obtained when the component solvents were combined beforehand and delivered with one pump. The apparent increase in efficiency was probably due once again to the slow drift in solvent strength when two pumps were used. In this case, the standard equations for plate height calculations under isocratic conditions may only have been good to a first approximation. However, the data presented in Fig. 4 do show that no loss in column efficiency was obtained when the components of the mobile phase were mixed with the combined mixing devices.
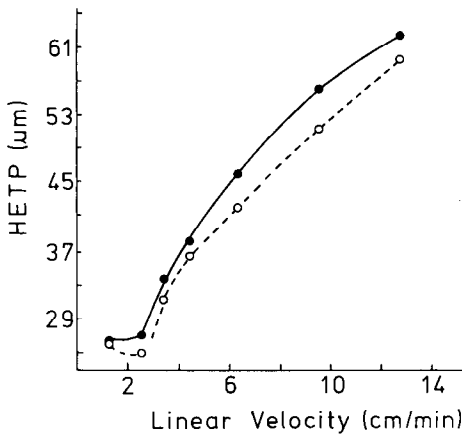
Fig. 4. Plots of plate height (for toluene) *vs.* linear velocity for premixed and on-line mixed methanol-water (70:30, v/v) mobile phases. (●) Premixed mobile phase; (○), mobile phase mixed on-line with combined mixing devices.

Computer simulations

The effects of flow-rates, ramp-rate and stirred mixing chamber volume upon the linearity of a gradient were studied by computer simulation. The model used for these simulations is described in the experimental section. Fig. 5 shows two simulated gradients in which the strong solvent concentration was increased from 0 to 100% at 2%/min. The simulated flow-rate was 20 μl/min. A perfectly linear gradient was obtained when a "zero-dead volume" mixing chamber was simulated. However, the initial and final portions of the gradient increased at a slower rate when the simulated mixing chamber had a volume of 100 μl; this was due to the effect of "exponential dilution" caused by the mixing chamber. The middle portion of the gradient was linear, and the concentration of strong solvent increased at the preprogrammed rate. The extent of distortion caused by exponential dilution can be quantitated through use of the concentration error (CE), which is labelled on Fig. 5; it is the maximum distance along the concentration axis between the gradients simulated for the zero and nonzero volume mixing chambers. In the example shown in Fig. 5, CE has a value of 10%.
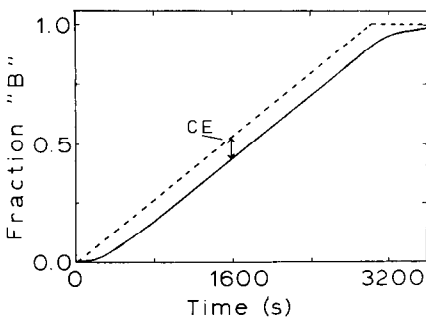

Fig. 5. Simulated gradients obtained with different mixing chamber volumes. (----) Zero-volume simulated mixing chamber; (——), 100-μl simulated mixing chamber.

The CE is a fairly useful parameter for describing the linearity of a gradient because its magnitude (in units of strong solvent concentration) is equal to the size of the intervals (in the same concentration units) at the beginning and end of the gradient during which the gradient will be non-linear. For example, if CE is 5%, a gradient that spans a strong solvent concentration range of 50 to 100% will not be linear from 50 to 55% and from 95 to 100% strong solvent. Note that the error is in absolute terms. The dependence of CE upon the flow-rate, ramp-rate, and mixing chamber volume was evaluated. It was found that CE is directly proportional to the ramp-rate and mixing chamber volume, and inversely proportional to the flow-rate. Further studies showed that CE can be expressed as a simple function of these parameters:

$$CE = \frac{\text{ramp-rate} \times \text{mixing chamber volume}}{\text{flow-rate}} \tag{2}$$

Eqn. 2 yields CE values that exactly match those obtained by simulation. It also gives CE values that compare well with those obtained experimentally with a methanol to acetone–methanol gradient using the 80-μl stirred mixing chamber described above. The ramp-rates and flow-rates were varied, and the comparisons are shown in Table I. The agreement in all cases is quite good, considering that the simulation does not take into account pumping errors and other such artifacts that arise at very low or very high strong solvent concentrations.

The implications of eqn. 2 are quite obvious. For example, it is well-known that a larger mixing chamber causes more gradient distortion since a larger volume must be flushed out in order to change the concentration of strong solvent in the mobile phase by a given amount. Higher ramp-rates are accompanied by more distortion of the linearity because of the finite amount of time needed to displace enough volume from the mixing chamber to obtain the desired change in concentration. Finally, the linearity is improved at higher flow-rates, because the mixing chamber is flushed out more quickly. In addition, the formula given in eqn. 2 allows easy quantitation of the effects of these variables.

Eqn. 2 is valid over the entire range of HPLC conditions if it is assumed that the linearity of the gradient is controlled primarily by exponential dilution and that the incoming solvents are perfectly mixed in the chamber. The formula would be

TABLE I

COMPARISONS OF CONCENTRATION ERROR VALUES OBTAINED FROM EQN. 2 AND EXPERIMENTALLY

The mixing chamber volume was 80 μl in each case.

Flow-rate (μl/min)	Ramp-rate (%/min)	Concentration error (%)	
		Formula	Experimental
30	1	2.7	2.9
30	3	8.0	8.3
50	2	3.2	2.5
50	5	8.0	7.9

useful for determining mixer volumes for microbore and capillary HPLC gradient systems. It also would be useful if gradient elution in fast HPLC were being considered.

Application to polystyrene separations

The gradient microbore system described above was used in the separation of low-molecular-weight polystyrene oligomers. A comparison of gradient runs in conventional and microbore HPLC is shown in Fig. 6. The components of the mobile phase, acetonitrile and water, were mixed with the combined low-volume mixing devices for microbore work, and with a 1-ml stirred mixing chamber for conventional HPLC runs. The linear velocity was the same in each case (6.36 cm/min), so the retention times for the microbore column were double those obtained for the conventional column, since the microbore column was twice as long. Partial resolution of the isomers of the $n = 3$ and higher oligomers was possible in both methods. The resolution of the trimer diastereomers was higher on the more efficient microbore column. However, the diastereomers of the higher-molecular-weight oligomers were resolved either to the same extent as obtained with the conventional column or to a slightly poorer extent. The solvent program reached 100% acetonitrile 40 min into
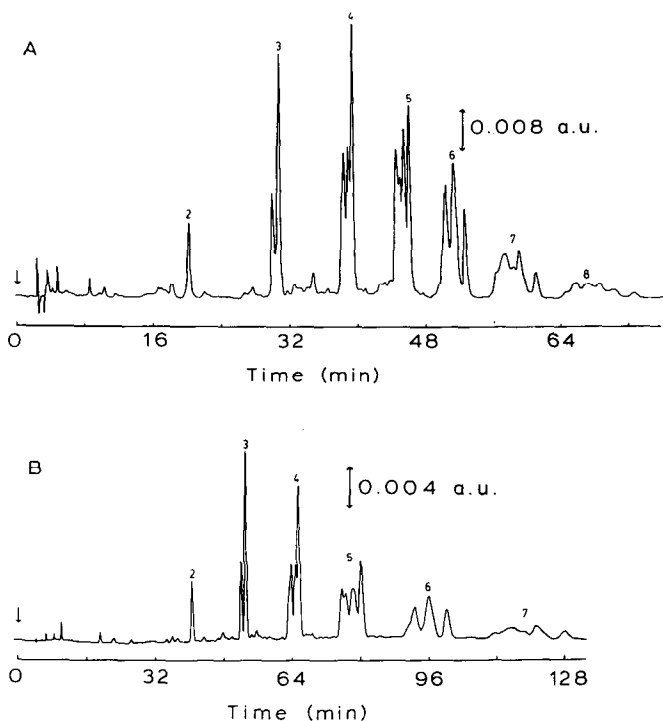

Fig. 6. Gradient separations of a MW 666 polystyrene sample. The number above each oligomer peak is the number of styrene units in the oligomer. Gradient: acetonitrile–water (80:20) to 100% acetonitrile at 0.5%/min. A, Conventional column (25 cm × 4.6 mm I.D.), 5-μm ODS-derivatized spherical silica (IBM Instruments), flow-rate = 1 ml/min, sample size = 25 μl. Detector set at 254 nm with 8-μl flow cell installed. B, microbore column (50 cm × 1 mm I.D.), 5-μm ODS-derivatized Zorbax (DuPont Instruments), flow-rate = 50 μl/min, sample size = 0.5 μl. Detector set at 254 nm with 0.5-μl flow cell installed.

the run. Therefore, the higher oligomers were retained on the microbore column for a longer time with pure acetonitrile as the mobile phase. The resolution of the isomers was thereby degraded somewhat, since water enhances this separation to some extent[27].

The homebuilt gradient microbore HPLC system performs at a level comparable to a conventional HPLC apparatus. The retention times of the major peaks had an average R.S.D. of 1.1% for conventional and of 1.4% for microbore HPLC. Variation of the initial mobile phase composition and the ramp-rate had the same effect for both methods. An increase in the initial percentage of acetonitrile greatly reduced the retention times of all oligomers. However, the resolution of the isomers was relatively insensitive to changes in the initial solvent composition. Increasing the ramp-rate did result in decreased resolution of the tetramer and pentamer diastereomers as well as decreased retention times. For the microbore column, a lower ramp-rate of 0.3% acetonitrile/min increased the resolution of those later isomer peaks to a level comparable to that obtained with the conventional column.

CONCLUSION

Thorough mixing of the mobile phase solvents is essential to achieve the maximum chromatographic performance from a microbore column. Poor mixing results in increased baseline noise, less reproducible retention times, and significant band broadening. The pulsed delivery of the syringe pumps used in this study necessitated the use of a stirred mixing chamber; static mixers did not yield satisfactory results. The major problem with dynamic mixers is that they do distort the linearity of the gradient. However, the formula developed from computer simulation results is useful for determining the conditions necessary to produce an acceptably linear gradient.

It is, of course, desirable to keep the volume of the stirred mixing chamber as low as possible. However, the mixer volume must be large enough to homogenize the pulses of solvent delivered to it. Gradient elution systems usually operate in cycles of some type, and it is generally agreed that the mixing chamber must be large enough to hold the volume delivered during two of these cycles. Therefore, a gradient system based on reciprocating pumps must have a high-pressure mixing chamber that is large enough to hold two stroke volumes. Any low-pressure gradient formers developed using solenoid proportioning valves should have a mixing chamber large enough to hold the volume delivered during two cycles of the valves. The solenoid valves would, therefore, have to be extremely fast in order to allow the mixing chamber volume to be low enough to avoid serious distortion of the gradient. Finally, the stepper motor-driven syringe pumps used in this study have a variable duty cycle period; it turns out that the minimum mixing chamber volume is only 11 μl for complete mixing from 5 to 95% of a 0 to 100% gradient at any flow-rate. Therefore, our 80-μl mixing chamber was larger than necessary, and a smaller volume could have been used if the situation had warranted it.

ACKNOWLEDGEMENTS

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